ILT4 REGULATES THE INVASION AND MIGRATION OF NSCLC CELLS BY REGULATING PI3K-AKT SIGNALING PATHWAY

Guowei Liu[1,#], Huanjuan Yang[2,#], Wenhua Liu[3], Chunchao Peng[4], Xiaoling Zheng[4], Husai Ma[1,*]
[1]Department of Thoracic Surgery, Qinghai Provincial People's Hospital, Xining 810007, Qinghai Province, China - [2]Department of Medical, Qinghai Provincial People's Hospital, Xining 810007, Qinghai Province, China - [3]Department of Nephrology, Qinghai Provincial People's Hospital, Xining 810007, Qinghai Province, China - [4]Department of Anesthesiology, Qinghai Provincial People's Hospital, Xining 810007, Qinghai Province, China
These authors contributed equally to this work as co-first author

ABSTRACT

Introduction: *To analyze the inhibitory effect of silencing immunoglobulin-like transcription factor 4 (ILT4) on invasion and migration of non-small cell lung cancer cells through positive regulation of phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signaling pathway.*

Methods: *40 cases of NSCLC tissues and adjacent normal tissues were collected from April 2019 to February 2020 in our hospital. The expression of ILT4 in non-small cell lung cancer (NSCLC) and adjacent normal tissues was detected by immunohistochemistry. A549 cells were cultured, and the si-ilt4 plasmid was transfected into A549 cells. After 4 hours of culture, the low expression group of ILT4 was obtained, and the control group was set up at the same time. Four multiple groups were set in each group. Transwell invasion assay was used to determine the invasion ability of the two groups. The cell migration ability of the two groups was measured by cell scratch test. The expression levels of ILT4, matrix metalloproteinase-2 (MMP-2), phosphatidylinositol 3 kinase (PI3K) and protein kinase B (Akt) in the two groups were measured by protein imprinting method.*

Results: *The expression of ILT4 in lung cancer tissues was significantly higher than that in adjacent normal tissues. Compared with the control group, the expression levels of ILT4, PI3K and Akt in the low expression group of ILT4 were significantly decreased (P<0.01). Compared with the control group, the invasion and migration ability of ILT4 low expression group was significantly decreased (P<0.01).*

Conclusion: *Silencing ILT4 expression may inhibit the invasion and migration of NSCLC cells by regulating the PI3K/Akt signaling pathway.*

Keywords: *ILT4, PI3K-AKT signaling pathway, non-small cell lung cancer, cell invasion, cell migration.*

DOI: 10.19193/0393-6384_2023_3_96

Received *March 15, 2022;* ***Accepted*** *January 20, 2023*

Introduction

Lung cancer is the most common malignant tumor originating from the bronchus or trachea in clinical practice. It can be divided into non-small cell lung cancer and small cell lung cancer according to the different pathological types. According to statistics, more than 85% of lung cancer patients are non-small cell lung cancer. At present, the main clinical treatment for lung cancer is surgical treatment, but surgical treatment has a high recurrence rate and poor prognosis[(1)]. Studies have found that the reasons for postoperative recurrence and poor prognosis of patients with Non-small cell lung cancer may be closely related to the invasion and migration of tumor cells[(2)]. Invasion and migration of cells are important links in the occurrence and development of Non-small cell lung cancer, and are also the most difficult problems in tumor treatment[(3)]. Immunoglobulin like transcription factor (ILT) belongs to the Immunoglobulin superfamily and is mainly expressed in natural killer cells, T cells and other cells, which can produce a wide range of immunomodulatory effects by conducting

inhibitory or activating signals[4]. ILT4, a member of the ILT family, can bind to the corresponding ligand to inhibit the activity of immune cells and natural killer cells. According to relevant reports, ILT4 is abnormally expressed in acute monocytic leukemia, breast cancer, colorectal cancer and other malignant tumors, and the level of deexpression is closely related to the degree of tumor malignancy[5]. However, its mechanism in NSCLC is still unclear.

In this study, the expression of ILT4 in NSCLC was firstly detected, and then the expression of ILT4 in NSCLC cell lines was silenced by siRNA interference technology to make ILT4 expression down-regulated, and the effect of ILT4 on the invasion and migration ability of NSCLC cells was detected, and its mechanism was explored and analyzed.

Data and methods

Experimental materials

A total of 40 cases of non-small cell lung cancer (NSCLC) tissues and normal adjacent tissues underwent surgical resection in hospital from April 2019 to February 2020 were collected. Non-small cell lung cancer cell line A549 (Wuhan Punosai Life Technology Co., LTD.).

Experimental reagents and instruments

Trypsin (Shanghai First Biochemical Pharmaceutical Co., LTD., Batch Number: 31022008, Specification: 2.5U); Hematoxylin Eosin (HE) dye (Shanghai Xinfan Biotechnology Co., LTD.); Immunohistochemical staining kit (Wuhan Moshak Biotechnology Co., LTD.); Fetal bovine serum (Shanghai Jianglin Biotechnology Co., LTD.); Real-time PCR kit [Thermo Fisher Scientific (China) Co., LTD.]; PI3K Antibody (Shanghai Hushang Biotechnology Co., LTD.); AKT Antibody [Sigma Aldrich (Shanghai) Trading Co., LTD.]; Paraffin slicer (Shenyang Hengsong Technology Co., LTD., Model: S7120); Carbon dioxide incubator (Shanghai Rundu Biological Technology Co., LTD., Model: Herocell 180); Optical microscope (Shanghai Yuguang Instrument Co., LTD., Model: XYU-60D); Low temperature high speed centrifuge (Shanghai Luxiang Yi Centrifuge Instrument Co., LTD., model: TGL-17M).

Experimental methods

• Cell culture: A549 cells were removed from the liquid nitrogen tank, melted and resuscitated in a 37°C water bath, the cell suspension in the frozen storage tube was sucked into a 10ml centrifuge tube fitted with an incubator for centrifuge, the supernatant was removed, and the cells were placed in a medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, and suspended and dispersed by blowing. Culture at 37°C and 5% CO_2 overnight. When the cell density in the culture flask reached about 85%, the subculture was carried out, 1ml trypsin was added for digestion for 3min, and the cells were gently blown to suspend, and incubated overnight in an incubator at 37°C with 5%CO_2.

• Cell transfection: A549 cells with good growth and logarithmic growth stage were taken for digestion and centrifugation, the supernatant was removed, and the cells were re-suspended in DMEM medium containing 10% fetal bovine serum. The cells were inoculated into 6-well plates with 10000 cells/well and cultured at 37°C in a 5%CO_2 incubator for 24h. Si-ILT4 plasmid was transfected into A549 cells. The medium was replaced with serum-free medium and continued to be cultured for 4h to obtain the ILT4 low expression group, while the control group was set. Configure four multiple groups for each group.

Observation indicators

The expression level of ILT4 in non-small cell lung cancer and adjacent normal tissues was determined by immunohistochemical method.

• Cell invasion: transwell invasion assay was used to determine the changes of cell invasion ability in both groups.

Transfected A549 cells were cultured in an incubator until the logarithmic growth phase, trypsin digested cells were added, beaten and mixed, and cell concentration was adjusted to 5×10^5 cells/mL. 200μL cell suspension of each group was absorbed and inoculated into the upper chamber of Transwell chamber. In the lower chamber, 600μL medium containing 10% fetal bovine serum was added, and incubated at 37°C in 5% CO_2 incubator for 24h, avoiding bubble formation during the process. The chamber was removed, the culture medium was removed, anhydrous ethanol was added, fixed at room temperature for 30min, and the residual liquid was absorbed. Stain with 0.1% crystal violet solution and gently wipe the attached cells above the microporous membrane of the upper compartment with a clean cotton swab. Use a microscope for observation.

• Cell migration: cell scratch test was used

to determine the changes of cell migration ability between the two groups. Transfected A549 cells were cultured and made into a single cell suspension, which was inoculated into a 6-well plate with 5×10^5 cells/well and cultured in a 5%CO_2 incubator at 37°C. When the cells grew to about 85%, a scratch was made with a sterile pipette gun, and photos were taken at 0h and 48h respectively. Repeated studies to reduce errors. ILT4, Matrix metalloproteinase-2 (MMP-2), Phosphatidylinositol 3 kinase were determined by protein imprinting method. PI3K, protein kinase B (AKT) expression level.

The A549 cells of each group were collected and lysed with lysate. The working solution was prepared according to the BOVINE serum albumin kit for protein quantification. According to the instructions, 5% concentrated adhesive, 12% separated adhesive, electrophoresis buffer, membrane transfer buffer, TBST buffer and 5%BSA sealing fluid; An electrophoresis system was installed for electrophoresis, electrophoresis gel separation, and target protein cutting; The separation glue was placed on the dePVDF film soaked in methanol and inserted into the transfer film in the transfer film tank; The PVDF membrane was removed and incubated with primary antibodies ILT4, MMP-2, PI3K and AKT. After incubation, the PVDF membrane was incubated at 4°C overnight.

The primary antibodies non-specifically bound to the membrane were washed by TBST, and the corresponding secondary antibodies were added and incubated at room temperature for 1-2h. It was developed in a gel imaging system.

Statistical methods

T test was used for comparison of measurement data in this study, expressed as ($\bar{x}\pm s$). SPSS18.0 software was used for statistical data analysis in this study, and $P<0.05$ was considered as statistically significant.

Results

Comparison of ILT4 expression levels in lung cancer tissues and adjacent normal tissues

The expression level of ILT4 in lung cancer tissues was significantly higher than that in normal adjacent tissues. As shown in Figure 1.

Comparison of cell invasion ability between the two groups

Compared with the control group, the invasion ability of ILT4 low expression group was significantly decreased ($P<0.01$). See Figure 2 and Table 1.

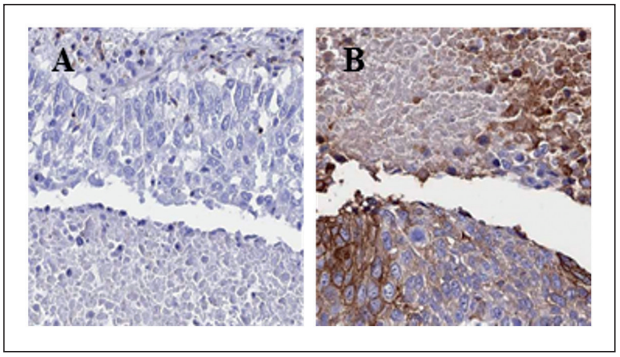

Figure 1: Comparison of ILT4 expression levels in lung cancer tissues and adjacent normal tissues.
Figure A: Normal adjacent tissue; Figure B: Lung cancer tissue.

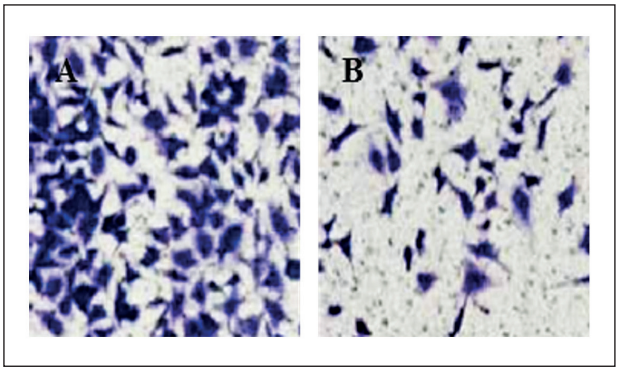

Figure 2: Invasion ability of the two groups of cells.
Figure A: Control group; Figure B: ILT4 low expression group.

Group	Number of invaded cells
ILT4 low expression group	94.76±14.76
Control group	188.66±24.76
t	6.515
P	0.001

Table 1: Comparison of cell invasion ability between the two groups ($\bar{x}\pm s$).

Comparison of cell migration ability between the two groups

Compared with the control group, the migration ability of ILT4 low expression group was significantly decreased ($P<0.01$). See Figure 3 and Table 2.

Comparison of the expression levels of ILT4, MMP-2, PI3K and AKT in the two groups

Compared with the control group, the expression levels of ILT4, MMP-2, PI3K and AKT in ILT4 low expression group were significantly decreased ($P<0.01$). See Figure 4 and Table 3.

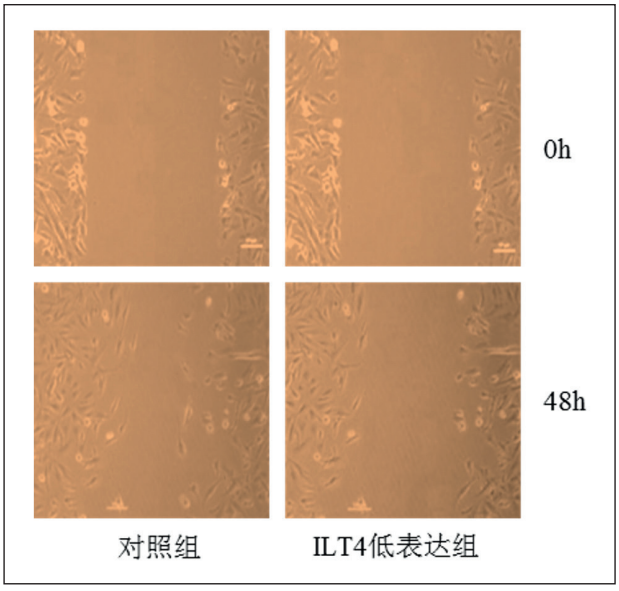

Figure 3: Cell migration capacity of the two groups.

Group	Migrating cell number
ILT4 low expression group	197.69±16.88
Control group	346.96±23.25
t	10.391
P	<0.001

Table 2: Comparison of cell invasion ability between the two groups ($\bar{x}\pm s$).

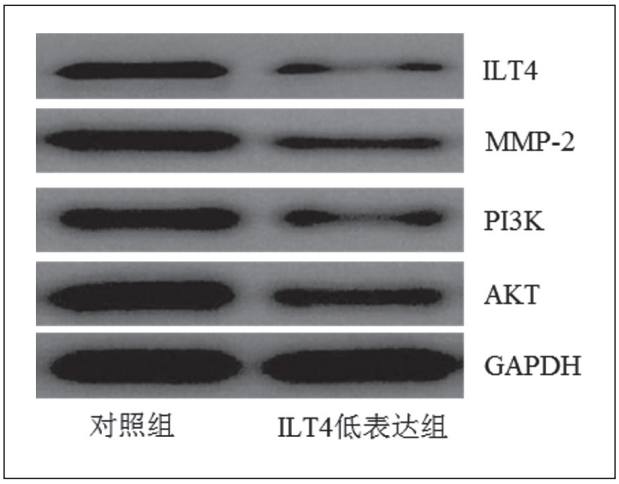

Figure 4: Expression levels of ILT4, MMP-2, PI3K and AKT in the two groups.

Group	ILT4	MMP-2	PI3K	PI3K
ILT4 low expression group	0.13±0.10	0.96±0.03	0.20±0.07	0.20±0.07
Control group	0.97±0.02	0.11±0.06	0.77±0.04	0.77±0.04
t	16.474	25.342	14.140	14.140
P	<0.001	<0.001	<0.001	<0.001

Table 3: Comparison of the expression levels of ILT4, MMP-2, PI3K and AKT in the two groups ($\bar{x}\pm s$).

Discussion

Lung cancer is the "number one killer" endangering human health at present, and its morbidity and mortality remain high, and the trend is increasing year by year. According to statistics, more than 2 million patients die of lung cancer worldwide every year, and lung cancer is the main cause of human cancer death[6]. According to the histopathology, lung cancer can be divided into small cell lung cancer and non-small cell lung cancer. Non-small cell lung cancer is the main pathological type of lung cancer, accounting for more than 80% of the total lung cancer, and more than 30% of patients at the time of initial diagnosis have advanced stage, the treatment effect and prognosis of patients are poor. Therefore, it is important to find the pathogenesis of NSCLC, explore its therapeutic targets, reduce the recurrence rate of patients, and improve the prognosis of patients.

ILT4 is an immunoglobulin, which is mainly transcribed in monocytes, T and B cells. It can bind to major histocompatibility complex through classical or non-classical methods to induce immunosuppressive T cells and inhibit the activation and proliferation of T cells, thus playing an immunosuppressive role[7]. The expression of ILT4 can be regulated by interleukin-10 (IL-10), Interferon-α (INF-α) and growth factors. Studies have confirmed that ILT4 is significantly expressed in breast cancer, leukemia and non-small cell lung cancer cells, and its level is significantly correlated with lymph node metastasis[8]. However, there are few reports about its related role in non-small cell lung cancer. The purpose of this study was to explore the effect of silencing ILT4 on invasion and migration of non-small cell lung cancer cells and the mechanism of action, so as to provide relevant data for clinical treatment. Invasion and metastasis of tumor is a complex process and an important cause of relapse in patients, which may involve many aspects, among which extracellular matrix has an important relationship with invasion and metastasis of tumor[9]. The extracellular matrix is a natural barrier during the invasion and diffusion of tumor cells, and the invasion and migration of tumor cells require the degradation of adjacent tissues' penetrating basement membrane and the extracellular matrix of vascular tissue cells[10]. Matrix metalloproteinase is a zinc-dependent endopeptidase, which can almost completely degrade the extracellular matrix and support apoptosis during inactivation, affecting the

survival of tumor cells[11]. MMP-2, a member of matrix metalloproteinases, plays an important role in cell invasion and migration. Studies have found that increased expression level of MMP-2 can cause damage to the continuity of the basement membrane, which may be the result of mMP-2's ability to degrade type IV collagen[12]. Some scholars have found that the level of MMP-2 in patients with different clinical stages is significantly different, and the more serious the disease, the higher the level[13]. MMP-2 can be used as a marker to judge the prognosis of patients. The results of this study showed that the invasion and migration of ILT4 cells were significantly weakened after silencing the expression of ILT4. ILT4 can promote the invasion and migration of cells.

PI3K/AKT signaling pathway is an important signal transduction pathway associated with oncogenes and a variety of receptors. Among them, PI3K is an important kinase of phosphatidylinositol, and its activation can produce a second messenger PIP3 on the plasma membrane, which is necessary for AKT to transfer to the cell membrane and be activated. Activated AKT phosphorylates to activate or inhibit its downstream target proteins, thereby playing an anti-apoptosis and proliferation role[14]. In the study of breast cancer, PI3K can mediate the invasion driven by integrin $\alpha 2\beta 1$, and the overexpression of AKT can increase the invasion and migration of cells by up-regulating integrin $\beta 1$ through type IV collagen. AKT negatively regulates the function of integrin $\alpha 2\beta 1$ in the disruption of c-ErBB2-induced matrix adhesion and formation[15]. These results suggest that the PI3K/AKT signaling pathway is involved in the invasion and migration of cells mediated by adhesion factors. The results of this study showed that silting ILT4 expression could significantly inhibit the expression of PI3K and AKT, and inhibit the activation of PI3K/AKT signaling pathway. This may be an important reason for the decrease of biological activity of A549 cells.

In conclusion, silencing ILT4 expression may inhibit the invasion and migration of NSCLC cells by regulating the PI3K/AKT signaling pathway.

References

1) Peters S, Camidge D R, Shaw A T, et al. Alectinib versus Crizotinib in Untreated ALK-Positive Non-Small-Cell Lung Cancer[J]. New England Journal of Medicine, 2017, 377(9): 829.
2) Soria J C, Ohe Y, Vansteenkiste J, et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer [J]. New England Journal of Medicine, 2018, 378(2): 113.
3) Zhang X, Li G, Guo Y, et al. Regulation of ezrin tension by S-nitrosylation mediates non-small cell lung cancer invasion and metastasis[J]. Theranostics, 2019, 9(9): 2555-2571.
4) Dhont L, Pintilie M, Kaufman E, et al. Helicase-like transcription factor expression is associated with a poor prognosis in Non-Small-Cell Lung Cancer (NSCLC)[J]. BMC Cancer, 2018, 18(1): 429.
5) Basilico C, Arnesano A, Galluzzo M, et al. A High Affinity Hepatocyte Growth Factor-binding Site in the Immunoglobulin-like Region of Met[J]. Journal of Biological Chemistry, 2018, 283(30): 21267-21277.
6) Soria J C, Tan D S W, Chiari R, et al. First-line ceritinib versus platinum-based chemotherapy in advanced ALK-rearranged non-small-cell lung cancer (ASCEND-4): a randomised, open-label, phase 3 study [J]. Lancet, 2017, 389(10072): 917-929.
7) Zhang P, Guo X, Li J, et al. Immunoglobulin-like transcript 4 promotes tumor progression and metastasis and up-regulates VEGF-C expression via ERK signaling pathway in non-small cell lung cancer[J]. Oncotarget, 2015, 6(15):13550.
[8] Minnich M, Tagoh H, Belt P, et al. Multifunctional role of the transcription factor Blimp-1 in coordinating plasma cell differentiation[J]. Nature Immunology, 2016, 17(3): 331-343.
9) Wei C H, Wu G, Cai Q, et al. Correction to: MicroRNA-330-3p promotes cell invasion and metastasis in non-small cell lung cancer through GRIA3 by activating MAPK/ERK signaling pathway[J]. Journal of Hematology & Oncology, 2018, 11(1): 6.
10) Lim S B, Tan S J, Lim W T, et al. An extracellular matrix-related prognostic and predictive indicator for early-stage non-small cell lung cancer[J]. Nature Communications, 2017, 8(1): 1734.
11) Fan S, Liao Y, Liu C, et al. Estrogen promotes tumor metastasis via estrogen receptor beta-mediated regulation of matrix-metalloproteinase-2 in non-small cell lung cancer[J]. Oncotarget, 2017, 8(34): 56443-56459.
12) Jiang L Y, Bi R, Ding F B, et al. Prognostic significance of overexpressed matrix metalloproteinase-2, mouse-double minute: 2 Homolog and epidermal growth factor receptor in non-small cell lung cancer[J]. Journal of B.u.on. Official Journal of the Balkan Union of Oncology, 2016, 21(2): 341.
13) Drzewiecki-Jędrzejczyk, Magdalena, Wlazeł, Rafał, Terlecki M, et al. Serum metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in lung carcinoma patients[J]. Journal of Thoracic Disease, 2017, 9(12): 5306-5313.
14) Zhen Y, Li D, Li W, et al. Reduced PDCD4 Expression Promotes Cell Growth Through PI3K/Akt Signaling in

Non-Small Cell Lung Cancer[J]. Oncology Research, 2016, 23(1): 61-68.

15) Zhu X H, Jiang H, Li J, et al. Anticancer Effects of Paris Saponins by Apoptosis and PI3K/AKT Pathway in Gefitinib-Resistant Non-Small Cell Lung Cancer[J]. Medical science Monitor International Medical Journal of Experimental & Clinical Research, 2016, 22(10): 1435-1441.

Corresponding Authors:
Husain Ma
Qinghai Provincial People's Hospital, 2 Gonghe Road, Chengdong District, Xining City, Qinghai Province, China
Email: 94528086@qq.com
(China)